Chapter 6

DNA copy number alterations mark disease progression in pediatric Chronic Myeloid Leukemia (CML)

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ABSTRACT

Early recognition of children with chronic phase chronic myeloid leukemia (CML-CP) at risk for developing a lymphoid blast crisis (LyBC) is desirable, because therapy options in CML-LyBC are limited. We used Multiplex Ligation-dependent Probe Amplification (MLPA) to determine whether B-cell lymphoid leukemia-specific copy number alterations (CNAs) (e.g. *IKZF1*, *PAX5*, and *CDKN2A* deletions) could be detected in CML-CP and may be used to predict disease progression to LyBC. CNAs were detected in all patients with CML-LyBC, but in none of the 77 patients with CML-CP. Based on this study we conclude that CNAs remain a hallmark of disease progression.

INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal myeloproliferative expansion of transformed, primitive hematopoietic progenitor cells. The *BCR-ABL1* fusion gene, resulting from the Philadelphia chromosome translocation *t*(9;22)(q34;q11), is found in up to 95% of all CML patients.[1] Clinical and laboratory studies demonstrate that the BCR-ABL1 fusion protein plays an essential role in the initiation, maintenance and progression of CML.[2]

With the introduction of tyrosine kinase inhibitors (TKIs), CML has transformed from a fatal disease to a leukemia subtype with a favourable prognosis.[2] Estimated progression-free survival rates at 36 months are 98% in children with chronic phase CML (CML-CP).[3-5] However, once a blast crisis (BC) has occurred, treatment options are limited with a median overall survival of approximately 5.3 months in adult lymphoid BC (CML-LyBC) patients.[6] Therefore, early recognition of patients at risk of developing a BC seems important.

There is accumulating evidence that specific gene abnormalities contribute to the transformation from CML-CP to CML-BC in adults.[7,8] In addition to the *BCR-ABL1* translocation *t*(9;22)(q34;q11), deletions in *IKZF1*, *PAX5*, and / or *CDKN2A* have been frequently reported in CML-LyBC.[7,8] Similar deletions in *IKZF1*, *PAX5*, and *CDKN2A* are also frequently observed in B-cell precursor acute lymphoblastic leukemia (BCP-ALL).[7,9-11]

In the present study we used Multiplex Ligation-dependent Probe Amplification (MLPA) analysis to screen for the presence of copy number alterations (CNAs) in a large cohort of 77 children with CML-CP, two patients with accelerated phase CML (CML-AP), and one patient with CML-LyBC, to investigate whether deletions in *IKZF1* and other genes are detectable in pediatric CML and if they could be used to predict disease progression in CML-CP.

MATERIAL AND METHODS

Patients

A total of 80 children with newly diagnosed CML were included: 51 patients from Germany and 29 patients from the Netherlands. Disease stage was determined at time of diagnosis following standard World Health Organization criteria (% blast cells in peripheral blood (PB) or bone marrow (BM), % blast cells plus promyelocytes in PB, % basophiles in PB, persistent thrombocytopenia unrelated to therapy and presence of extra medullary blast involvement).

DNA isolation

Genomic DNA was isolated according to manufacturer's protocol from whole peripheral blood or bone marrow using the QIAamp DNA Blood Mini Kit or from mononuclear cells using the QIAamp DNA easy kit (Qiagen, Hilden, Germany) for the German and Dutch samples, respectively. All isolated DNA was quantified by NanoDrop spectrophotometry (NanoDrop, Wilmington, DE, USA).

Multiplex Ligation-Dependent Probe Amplification (MLPA)

Targeted copy number screening of eight selected loci (*IKZF1*, *BTG1*, *CDKN2A/B*, *EBF1*, *ETV6*, *PAX5*, *RB1*, and the PAR1 region) was performed in the cohort by means of MLPA using the P335-B2 SALSA MLPA kit (MRC-Holland, Amsterdam, The Netherlands) as described previously.[7,9-11] All samples contained >50% *BCR-ABL1* positive cells, as MLPA analysis is sufficient to detect copy number alterations when they are present in more than 50% of the cells (MRC-Holland).

Direct sequencing for detection of point mutations in *IKZF1* gene

To search for *IKZF1* point mutations, direct sequencing was performed on material from patients who experienced a blast crisis but were negative for *IKZF1* deletions by MLPA. Primers for polymerase chain reaction (PCR) reactions were designed in all coding regions of *IKZF1*. The sequences of designed primers are listed in Supplementary Table 1. Amplification was carried out using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). After the PCR products were verified by electrophoresis, direct DNA sequencing was performed on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Sequences were analyzed using Vector NTI® software (Life Technologies, Carlsbad, CA, USA) and aligned against a reference sequence obtained from USCS Genome Bioinformatics (NM_006060) (University of California, Santa Cruz, CA, USA).

RESULTS

A cohort of 80 children with newly diagnosed CML was analyzed. Patients' characteristics are listed in Supplementary Table 2. Of the 80 patients, 77 patients were diagnosed with CML-CP, 2 with CML-AP, and 1 with CML-LyBC. Four patients diagnosed with CML-CP experienced progression to a BC (two myeloid BC (MyBC) and two LyBC). Both patients diagnosed with CML-AP (Patients 58 and 67) experienced progression to LyBC 6.5 and 8.1 months after diagnosis, respectively. Patient 22, diagnosed with CML-LyBC, experienced a LyBC relapse.

CNAs in *BTG1*, *CDKN2A/B*, *EBF1*, *ETV6*, *IKZF1*, *PAX5*, and *RB1* were determined using MLPA analysis. An *IKZF1* deletion involving exons 4 to 7 (del 4-7) was identified in one patient diagnosed with CML-AP (Patient 58, Table 1). In the patient diagnosed with CML-LyBC an *IKZF1* del 4-7 and an *EBF1* deletion were identified (Patient 22, Table 1). No CNAs were identified in the 77 patients diagnosed with CML-CP (Table 1). Patients who experienced disease progression were further analyzed. There was no material available for the two patients that progressed to MyBC at this time point. In all of the four patients that showed progression to LyBC, CNAs were detected at BC (Table 2). Patient 62, diagnosed with CML-CP, experienced LyBC 4.1 months after diagnosis. At time of LyBC, a new *IKZF1* del 1-8 and a *PAX5* deletion were detected (Table 2). Patient 64, diagnosed with CML-CP, experienced LyBC 5.4 months after diagnosis. At time of LyBC, a new *CDKN2A/B* deletion was detected (Table 2). Sanger sequencing was performed, screening all exons of *IKZF1* using material from patients 18 and 32, at time of CML-CP, and patient 64, at LyBC onset. However, no *IKZF1* mutations were found. Both patients diagnosed with CML-AP experienced LyBC at 6.5 and 8.1 months after diagnosis, respectively. An *IKZF1* deletion encompassing exons 4-7 was found in patient 58 at time of diagnosis (Table 2); at time of LyBC, this *IKZF1* 4-7 deletion, but no other CNAs were found. No CNAs or point mutations could be detected in patient 67 at time of diagnosis, although at time of LyBC, novel *IKZF1* del 1-8 and *PAX5* deletions were detected (Table 2).

Table 1 Copy number alterations detected in pediatric CML at diagnosis

Disease stage at diagnosis	**Gene** ***IKZF1, CDKN2A/B, PAX5, ETV6, BTG1, RB1, CRLF2, EBF1***
Chronic phase	
N = 77	None
Accelerated phase	
Patient 58	*IKZF1* del 4-7
Patient 67	None
Lymphoid blast crisis	
Patient 22	*IKZF1* del 4-7, *EBF1* del

Table 2 Copy number alterations detected in CML patients with disease progression

Patient	Disease stage	Time of progression (months after diagnosis)	Copy number alterations
18	CP		No CNAs
	MyBC	7.8	No material available
32	CP		No CNAs
	MyBC	5.7	No material available
58	AP		*IKZF1* del 4-7
	LyBC	6.5	*IKZF1* del 4-7
62	CP		No CNAs
	LyBC	4.1	*IKZF1* del 1-8, *PAX5*
64	CP		No CNAs
	LyBC	5.4	*CDKN2A/B*
67	AP		No CNAs
	LyBC	8.1	*IKZF1* del 1-8, *PAX5*

Abbreviations: CML= chronic myeloid leukemia; CP= chronic phase; MyBC= myeloid blast crisis; LyBC= lymphoid blast crisis; AP= accelerated phase; CNA= copy number alterations.

DISCUSSION

This study characterized copy number alterations using MLPA analysis in the largest pediatric CML cohort to date. MLPA analysis was used to screen for deletions in eight different genes that are frequently deleted in CML-LyBC and BCP-ALL.[7,9-11] Copy number alterations were detectable in material from one of the CML-AP and in all of the CML-LyBC patients, while no CNAs were found in any of the 77 CML-CP samples.

The samples from all of the 77 analyzed CML-CP cases contained ≥63% *BCR-ABL1* positive cells, which should allow detection of clonal deletions by MLPA. CNAs were restricted to patients experiencing progressive disease. In one patient, presenting with a CML-AP, an *IKZF1* exon 4-7 deletion was detected at time of diagnosis. Recurrent deletions at time of CML-LyBC were found in *IKZF1*, *PAX5*, and the *CDKN2A/B* locus. Recently, a comparable study was performed on a smaller cohort of mainly adult CML patients (*N* = 39, 30 CML-CP (3 pediatric and 27 adult patients) and 9 CML-LyBC (all adults)).[8] In this study CNAs were found in material of only two imatinib-resistant adult patients at time of CML-CP, but several CNAs were detected during CML-LyBC.[8]

Our results in pediatric CML are also in accordance with studies performed in adults.[7,12-14] Mullighan *et al* used SNP arrays on 34 adult CML cases and described a mean of 0.47 CNAs per CML-CP whereas in CML-AP and CML-LyBC the mean was 1.14 and 7.8, respectively.[7] In a more recent study, no *IKZF1* deletions were detected in CML-CP or CML-AP (*N* = 104).[14] Therefore, we conclude that clonal CNAs are rare or even absent in CML-CP, but are relatively common at progressed stages, which is consistent with the notion that the BCR-ABL1 fusion protein is sufficient to induce CML, but additional genomic changes are required for disease progression.[7,12-14]

In vitro long term cultures suggest that loss of *IKZF1* and the presence of the BCR-ABL1 fusion protein synergistically contribute to leukemogenesis, resulting in aggressive lymphoid leukemogenesis as observed in CML-LyBC.[15] *IKZF1*, *PAX5*, and *CDKN2A* deletions are also recurrently found in both diagnosis and relapsed BCP-ALL, as shown previously.[9,10] Our results not only support the notion that BCR-ABL1 synergizes with these specific genetic events, but also suggest that there are intriguing similarities in the development of CML-LyBC and relapsed BCP-ALL. In addition, based on the type of CNAs found both in children and adults with CML, there appear to be no large changes in disease mechanism with age.

In summary, we conclude that, using MLPA analysis, clonal CNAs could not be detected in pediatric CML-CP but remain a hallmark of disease progression.

SUPPLEMENTARY INFORMATION

Supplementary Table 1 Primers used for amplification and direct sequencing of *IKZF1* gene

Primers	Sequence
IKZF1_x1_F	TGTAAAACGACGGCCAGTGGCCAAGTTAGCAGGACACTC
IKZF1_x1_R	CAGGAAACAGCTATGACCGGGTCTACCAACCTTACCGC
IKZF1_x2_F	TGTAAAACGACGGCCAGTCCTTGTTGTTAAATAGCATAGGGG
IKZF1_x2_R	CAGGAAACAGCTATGACCACCAAGCACTGTGACTTCCG
IKZF1_x3_F	TGTAAAACGACGGCCAGTCCTCATGCCACCCTCTCAAG
IKZF1_x3_R	CAGGAAACAGCTATGACCTGCATCCCTTCATCACTGTC
IKZF1_x3b_F	TGTAAAACGACGGCCAGTGAGTAGCTGAACAGTGGTTTTGAG
IKZF1_x3b_R	CAGGAAACAGCTATGACCGTTGGTGACAGAAAATATGGC
IKZF1_x4_F	TGTAAAACGACGGCCAGTTTTGCTGCTGTGTTGTTTTG
IKZF1_x4_R	CAGGAAACAGCTATGACCTGCTTTCCTCCTTCAAACCC
IKZF1_x5_F	TGTAAAACGACGGCCAGTCGTGGGAAACAACTTTCTCG
IKZF1_x5_R	CAGGAAACAGCTATGACCCAGAGTGGAGGAATCCCG
IKZF1_x6_F	TGTAAAACGACGGCCAGTATTGCATGCATTCCCCTTAC
IKZF1_x6_R	CAGGAAACAGCTATGACCCTCCTTCCCCACCGTGC
IKZF1_x7_F	TGTAAAACGACGGCCAGTTTTAACATTGGACGCGACTG
IKZF1_x7_R	CAGGAAACAGCTATGACCCCCTCAACTCATTTCTACTTGC
IKZF1_x8_1_F	TGTAAAACGACGGCCAGTCCAGACCTGACCGGTTCC
IKZF1_x8_1_R	CAGGAAACAGCTATGACCCTCCTCCTTGAGCGACAGC
IKZF1_x8_2_F	TGTAAAACGACGGCCAGTAACAGCTGCCAAGACTCCAC
IKZF1_x8_2_R	CAGGAAACAGCTATGACCCAGTCTATGCTGCTGGCG

Supplementary Table 2 Patients' characteristics

Demographics of clinical characteristics	No. of patients (*N* = 80)	%
Follow up, months median (range)	31.5 (0.6-231.1)	
Age, years		
Median (range)	11.1 (1.2-18.0)	
= 4	5	6.3
5-9	26	32.5
10-14	30	37.5
15-19	19	23.8
Sex		
Male	45	56.3
Female	35	43.8
Disease stage		
Chronic phase	77	96.3
Accelerated phase	2	2.5
Lymphoid blast crisis	1	1.3
Myeloid blast crisis	0	0.0

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